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Introduction

Vitamin a derivatives (retinoids) are known to be potent regulators of cell proliferation and epithelial cell differentiation and specifically inhibit the growth of several cancers. The mechanisms of action of retinoids are just starting to be understood. For example, retinoids are known to inhibit the function of AP-1, a transcription factor complex that is involved in cell proliferation and neoplastic transformation. Retinoids are also known to stabilize components of the adherens junction, the function of which is essential in preventing tumor progression and invasion. Previously, we have shown that in a breast cancer cell line, 9-*cis* retinoic acid induces a dramatic change in cell morphology, an increase in cell-cell adhesion strength, a decrease in cell proliferation, and an increase in the expression of the adherens junction molecule, β-catenin. This work now demonstrates that retinoic acid also induces the expression of a cadherin and that the expression of a cadherin, not of β-catenin is necessary and sufficient to mimic the effects induced by retinoic acid. Furthermore, this work also gives strong evidence to suggest that retinoic acid inhibits the β-catenin mediated cell signaling pathway, although in a manner independent of cadherin function.

Body

Background

Retinoids are important regulators of cell proliferation and epithelial differentiation, and can act as potent antitumor agents. For example, retinoids inhibit the growth of several human cancers, including melanoma, as well as colon and prostate cancer (6,11,15,19,29). In addition, retinoids inhibit growth and induce differentiation of breast cancer cells. For example, Anzano, *et al* showed that 9-*cis* RA reduced tumor incidence, average number of tumors and average tumor burden in a rat breast cancer model (2). Fontana, *et al* showed that 9-*cis*-RA also inhibits the growth of estrogen-receptor (ER) (+) breast cancer cell lines (16,35). We and others have demonstrated that the effects of retinoids may be mediated in part by the adherens junction (AJ) (9,13,41). The AJ is a molecular complex that is essential for initiating and maintaining strong cell-cell adhesion in epithelial cells (7). The AJ consists of a transmembrane cadherin molecule, the cytoplasmic catenins, and various cytoskeletal components (22). Cadherins are calcium dependent cell adhesion molecules that are involved organization of the developing embryo, and are essential for the maintenance of tissue integrity (39). Loss of cadherin function is well correlated with the progression of tumors to a more invasive phenotype (40). Loss of function may be due to a loss of expression of the normal gene which in epithelial cells is typically E-cadherin (40). However, expression of other cadherins have proven to be sufficient to inhibit the invasive phenotype and thus compensate for a loss of E-cadherin (32,36,37). Loss of cadherin function may also be due to changes that inhibit the inhibit a normal interaction with the catenins, and thus the actin cytoskeleton (40). The catenins (α, β, and γ) are essential for strong cell-cell adhesion (22). For example, loss of α- or β-catenin protein expression can disrupt normal cell-cell adhesion (5,31).

Loss of AJ function may also be mediated by activity of the transcription factor complex, AP-1. AP-1 is made up of the protooncogenes jun and fos, and its activity is associated with cell proliferation and neoplastic transformation (1). Fialka, *et al* showed that activation of c-jun in mammary epithelial cells resulted in a loss of epithelial polarity, a disruption of intercellular junctions and normal barrier function, and the formation of irregular multilayers. This was accompanied by a reduction in the association between E-cadherin and β-catenin in a manner that is independent of tyrosine phosphorylation (13).

Previously we have shown that retinoids have a profound effect on cell-cell adhesion (9). In the breast cancer cell line, SK-BR-3, RA induces a dramatic epithelialization that is accompanied by an increase in cell-cell adhesion strength, decreased cell proliferation, and an increase in the expression and triton insolubility of β-catenin. Our preliminary results have shown that RA induces the expression of an unknown cadherin. Our attempts to identify this cadherin, as well as our examination of the effect of retinoids on the members of the AJ are summarized below.

Our research has also led us into the area of cell signaling mediated by components of the adherens junction. While membrane-associated β-catenin acts as a component of the adherens junction, the cytoplasmic/nuclear pool of β-catenin is able to function in cell signaling. Evidence for the role of cytoplasmic/nuclear β-catenin as a signaling molecule comes from three observations: First, β-catenin is a member of the Wnt signaling pathway, and Wnt signaling results in increases in cytoplasmic and nuclear β-catenin; also, β-catenin associates with the product of the tumor suppresser gene, APC; finally, β-catenin interacts with and is essential for the transactivation function of members of the TCF/LEF family of transcription factors.

Both oncogenic and developmental effects of β-catenin are thought to result from an accumulation of β-catenin in the cytoplasm and nucleus (17). The downstream effects appear to be mediated by the interaction of β-catenin with members of the TCF/LEF family of transcription factors (3,26,27). T-cell factor (TCF) and lymphocyte enhancer binding factor (LEF) are members of a family of transcription factors that contain a DNA binding sequence homologous to the high mobility group (HMG) motif (4). These transcription factors have the capacity to induce distinct architectural changes in the DNA helix that, upon association with other proteins enable the formation of higher order nucleoprotein complexes (25). Interaction of β-catenin with TCF/LEF facilitates DNA bending to enable transcription of Wnt-induced genes (3). Thus, β-catenin/TCF/LEF complexes are thought to be the downstream effectors of the Wnt signal (23). For example, overexpression of LEF-1 in *Xenopus* is also able to mimic the effects of Wnt signaling and/or β-catenin overexpression (23). However, TCF/LEF bound alone to DNA acts to repress transcription (4). Therefore, transactivation is dependent upon and greatly enhanced by the association of TCF/LEF with β-catenin (23). Significantly, removal of the β-catenin binding domain from XTCF-1 inhibits both nuclear accumulation of β-catenin and β-catenin/Wnt signaling (23).

Because retinoids are important regulators of development and have potent anti-tumor effects, the role of retinoids in the regulation of cytoplasmic β-catenin and β-catenin/LEF signaling has now been examined.

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Summary of Results

SK-BR-3 cells have a homozygous deletion a significant portion of the E-cadherin gene (33). However, they exhibit strong calcium dependent cell adhesion upon retinoid treatment (9). In addition, SK-BR-3 cells express a membrane-associated protein that cross-reacts with a polyclonal antibody to the extracellular domain of E-cadherin (2). These observations strongly suggest that SK-BR-3 cells express an unknown cadherin that has some similarity to E-cadherin. In order to determine the effects of retinoids on the expression of this cadherin, a polyclonal pan-cadherin antibody was used. This antibody was raised against a region of the highly conserved C-terminal tail of cadherins, a region that also contains the β-catenin binding site. This antibody recognizes most cadherins except, notably E-cadherin (18,34). Immunocytochemistry was performed on SK-BR-3 cells +/- RA using this antibody. RA dramatically increased levels of this cadherin, the majority of which was found at the cell membrane. Notably, successful staining could only be achieved if the cells were fixed, then microwaved in citrate buffer in a process known as antigen retrieval. This requirement suggests that the epitopes of the pan-cadherin antibody are being masked by some other bound protein, and only gentle dissociation of the cadherin and this protein reveals those epitopes. Presumably, this bound protein is β-catenin.

Not surprisingly, RA also increased the expression of the cadherin solely in the NP-40 soluble and insoluble pools. In order to confirm that this cadherin was associating with other components of the AJ, immunoprecipitations were performed. β-catenin and separately α-catenin were immunoprecipitated from NP-40 lysates of SK-BR-3 cells +/- RA. Probing for cadherin expression revealed that both β-catenin and α-catenin associate with this cadherin. Furthermore, the level of cadherin that was coimmunoprecipitated paralleled the level of β-catenin and α-catenin, suggesting that retinoids did not necessarily increase the levels of cadherins or catenins independent of one another. α-catenin binding to β-catenin and plakoglobin also increase upon RA treatment, further confirming that all components of the AJ had been brought together in cells treated with RA.

Considering the relevance of a cadherin whose expression could potentially be regulated by RA, it seemed important to identify this unknown cadherin. However, efforts proved unsuccessful. SK-BR-3 cells do not express E-, N-, P-, or LI-cadherins, nor cadherins-6 or −11 as examined by western blot and/or RT-PCR. Therefore, in an attempt to amplify a region of the cadherin that is highly conserved among the various cadherins, degenerate primers were used for RT-PCR. Suzuki's group first used this method, and several groups have since used it to identify a number of cadherins (28,36-38). Using Suzuki's primers, an approximately 160 bp band could be amplified from melanoma cell RNA. This band presumably represents a portion of the mRNA encoding N-cadherin since melanoma cells are known to express N-cadherin (20). Suzuki and others have also attempted to identify the other bands amplified by this method, and these are generally believed to represent non-specific, or at least non-cadherin mRNAs (38). When these primers were used with SK-BR-3 RNA, a 160 bp band could not be

identified despite the successful amplification of a portion of the β-actin mRNA. Thus, the cadherin still remains unidentified.

In order to determine whether increased expression of β-catenin or cadherin was sufficient to mimic the effects of RA, these cells were transfected with β-catenin and E-cadherin expression vectors. SK-BR-3 cells overexpressing wild type β-catenin did not undergo a morphological change, nor did they express increased amounts of the endogenous cadherin. Expression of a mutant β-catenin construct, S37A β-catenin which is more resistant to degradation also had no effect (30). Interestingly, when SK-BR-3 cells are treated with RA, β-catenin levels increase predominantly at the membrane, but in cells overexpressing β-catenin, the β-catenin was found predominantly in the cytoplasm and nucleus suggesting that RA induces the capacity to sequester β-catenin to the membrane, which could occur through the increased expression of a cadherin.

In contrast to β-catenin, overexpression of E-cadherin did indeed result in an increase in β-catenin staining at the cell membrane, and an overall increase in β-catenin protein levels. In addition, when cells that were expressing the E-cadherin construct were selected by FACS and replated, these cells had the same morphology as RA-treated cells, suggesting that cadherin expression alone was sufficient to mimic the effects of RA on cell morphology. Cells expressing β-catenin and sorted in the same way exhibited no change in cell morphology. In order to determine whether cadherin expression was also necessary for the action of retinoids, two constructs were used. The first, δ35 E-cadherin encoded an E-cadherin molecule that lacked only the C-terminal 35 amino acids, which includes the β-catenin binding domain. I had anticipated that this construct would prevent the formation of a mature AJ because it would be unable to recruit the catenins and actin to the cadherin cluster, thus mediating only a weak cell-cell adhesion that would overwhelm the strong cell-cell adhesion mediated by the endogenous cadherin. The second construct, IL-2R E-cadherin is a chimera of the interleukin-2 receptor extracellular and transmembrane domains, and the entire E-cadherin cytoplasmic domain. I had anticipated that this construct would prevent the formation of a mature AJ by sequestering all available β-catenin to a non-adhesive molecule. Indeed, immunocytochemistry and western blot revealed that the δ35 E-cadherin construct was unable to increase β-catenin protein levels, while the IL-2R construct was able to do so very well. However, in both cases, expression of these anticipated "dominant-negative" constructs failed to inhibit the effects of RA on cell morphology and the recruitment of β-catenin to sites of cell-cell contact. The δ35 E-cadherin construct was unable to act as a dominant-negative most likely because it is unable to interfere with the homotypic binding of the endogenous cadherin. Surprisingly, the IL-2R E-cadherin construct also failed to act as a dominant-negative, suggesting that β-catenin are high enough to saturate both the IL-2R E-cadherin construct, and the endogenous cadherin.

Nevertheless, we have previously shown that the effects of RA on cell morphology and recruitment of β-catenin to sites of cell-cell contact are absolutely dependent upon extracellular calcium (9). Thus, it is very likely that cadherin expression is necessary to mediate the effects of RA on the AJ.

Next, the role of AP-1 in modulating the effects of RA was examined. AP-1 activity can result in cell proliferation and neoplastic transformation, and, more

specifically, AP-1 activity can disrupt the AJ by decreasing the association between β-catenin and E-cadherin (1,13). RA inhibits AP-1, and so we sought to determine whether inhibition of AP-1 alone could mimic the effects of RA on cell morphology and cadherin and β-catenin expression. First, using an AP-1 responsive reporter gene construct, it was confirmed that RA can indeed inhibit AP-1 activity. Next, in order to specifically inhibit AP-1 activity, a well characterized dominant-negative c-jun construct, TAM-67 was used (8). This construct can indeed inhibit AP-1 activity in SK-BR-3 cells. However, double labeling immunocytochemistry revealed that expression of TAM-67 in these cells had no effect on the expression or distribution of β-catenin or the cadherin, despite the high level of expression of TAM-67. Likewise, overexpression of c-jun, which induces retinoid resistance, had no effect on retinoid treated cells (42). Thus, inhibition of AP-1 seems neither sufficient nor necessary for the effects of RA on cell morphology and AJ stability. It should be noted, however, that in some cell types, the ability of retinoids to inhibit cell proliferation is due to their anti-AP-1 activity, while in others cell types this may not be the case ((10,12,21,24), and Powell Brown, personal communication). Nevertheless, the beneficial effects of retinoids may come from the ability of retinoids to inhibit AP-1 activity, thus inhibiting cell proliferation, as well as from the effects of retinoids on the AJ, which are presumably mediated by a cascade of events initiated by RARE-activation.

As our research led us to examine the effects of RA on cell signaling mediated by β-catenin, we first examined the effects of RA on cytomplasmic pools of β-catenin. In order to determine the effect of RA on cytoplasmic β-catenin, SK-BR-3 cells were transfected with a wild type β-catenin construct (30). Three pool fractionation revealed that the transfected β-catenin accumulated mostly in the cytoplasm and only partly at the membrane. Treatment of β-catenin-transfected cells with RA dramatically decreased levels of cytoplasmic β-catenin, while it increased levels at the membrane. RA also decreased levels of cytoplasmic β-catenin in other cells lines which constituitively express large amounts of β-catenin. Finally, the RA-mediated decrease in cytoplasmic β-catenin occurs only in the presence of calcium, suggesting that the RA-mediated decrease may require cadherin function.

In order to determine the effect of RA on β-catenin/LEF signaling, LEF-reporter assays were performed. SK-BR-3, MCF-7, and HS578T cells were transfected with an LEF-responsive luciferase reporter construct (SK-BR-3 cells were cotransfected with wild type β-catenin), and grown +/- RA for 48 hours. In all three cell lines, RA decreased LEF-reporter activity. Significantly, RA had also reduced levels of cytoplasmic β-catenin in these three cell lines as well. Surprisingly, RA was also able to reduce LEF-reporter activity in three RA-responsive cell lines, T47D (a modest reduction), ZR-75-B, and CaCo-2 in which there was no corresponding reduction in cytoplasmic β-catenin (14). To determine whether cadherin function is necessary to mediate the RA-induced reduction in LEF-reporter activity, SK-BR-3 cells were transfected with the LEF-responsive reporter and β-catenin and grown in low calcium medium. Surprisingly, RA is still able to reduce LEF-reporter activity even in low calcium medium. These results suggest that while the RA-mediated reduction in cytoplasmic β-catenin may be cadherin dependent, the reduction in β-catenin/LEF signaling is not.

Conclusions and Discussion

These data show that RA increases the expression of a β-catenin binding cadherin that mediates strong cell-cell adhesion. In addition, the expression and adhesive function of this cadherin is sufficient to mediate the effects of RA on morphology and recruitment of β-catenin to the cell membrane. However, neither the overexpression of β-catenin alone, nor the inhibition of AP-1 are able to mimic the RA-mediated effects. Thus, examining the effects of RA on this cell line has revealed that while RA can dramatically effect the function of the adherens junction, this effect may depend upon the increased expression and function of an unidentified cadherin. Therefore this cadherin may be critical in meditating the overall effects of retinoids as antiproliferative, differentiating chemotherapeutic agents.

With regards to β-catenin-mediated cell signaling, RA reduces the levels of cytoplasmic β-catenin, which has been regarded as the signaling pool of β-catenin, in a manner that is likely cadherin-dependent. Though RA induces a corresponding decrease in LEF-reporter activity, this decrease is not cadherin-dependent. This reveals the possibility of direct interactions between the cytoplamic/nuclear mediators of RA signaling and those of β-catenin/LEF signaling.

Appendices

Key Research Accomplishments
- The retinoid-induced cadherin was not identified as any of the several known cadherins, as demonstrated by western blot and RT-PCR.
- The retinoid-induced cadherin could not be isolated by degenerative RT-PCR, and thus remains unidentified.
- Retinoic acid increases the levels of membrane associated α-, β-, and γ-catenin.
- Neither the overexpression of β-catenin, nor the inhibition of AP-1 was sufficient to mimic the effects of retinoic acid on cell morphology and cell-cell adhesion.
- Expression of E-cadherin was necessary and sufficient to mimic the effects of retinoic acid on cell morphology and cell-cell adhesion.
- This suggests that the unidentified cadherin may be critical in mediating the effects of retinoic acid.
- RA reduces the levels of cytoplasmic β-catenin in a calcium (cadherin)-dependent manner
- RA induces a corresponding decrease in LEF-reporter activity, but this decrease is not cadherin-dependent.
- This reveals the possibility of direct interactions between the cytoplamic/nuclear mediators of RA signaling and those of β-catenin/LEF signaling.

Manuscripts
1) The Role of Cadherin, β-catenin, and C-jun in Retinoid-regulated Differentiation and TCF Signaling. Michael J. Pishvaian, Powell H. Brown, and Stephen W. Byers. (M anuscript in preparation).

2) Cross-Regulation of β-catenin-LEF/TCF and Retinoid Signaling Pathways. Vijayasurian Easwaran, Michael Pishvaian, Salimuddin, and Stephen Byers. *Current Biology* **9 (23)**: 1415-18. 1999.

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